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19 NOV 1997

21NOV97 E318924-1 D02029

Your Reference: B45122

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1 Please give the title of the invention: Novel Compounds

❷ Applicant's details

☐ **First or only applicant**

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Corporate Name: SmithKline Biologicals S.A.

19 NOV 1997

Country *(and State of incorporation, if appropriate)*: Belgium

2b If you are applying as an individual or one of a partnership please give in full:

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2c **In all cases,** please given the following details:

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2d, 2e and 2f: If there are further applicants please provide details on a separate sheet of paper

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3a Have you appointed an agent to deal with your application?

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❶

please give details below

Agent's name **West, Vivien**
Agent's address: CORPORATE INTELLECTUAL PROPERTY
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Agent's ADP number 4385316004

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4. Agent's or applicant's reference number *(if applicable)*

Claiming an earlier application date

5. Are you claiming that this application be treated as having been filed on the date of filing of an earlier application?

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☐ filing date *(day month year)*

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15(4) (Divisional) ☐ 8(3) ☐ 12(6) ☐ 37(4) ☐

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If you are declaring priority from a PCT Application please enter 'PCT' as the country and enter the country code (for example, GB) as part of the application number

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❼

applicant is not an inventor
re is an inventor who is not
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Inventorship

7. Are you (the applicant or applicants) the sole inventor or the joint inventors?
Please mark correct box
Yes ☐ No ☒ ➲ ***A Statement of Inventorship on Patents form 7/77 will need to be filed (see Rule 15).).***

❽ *Please supply duplicates of claim(s), abstract, description and drawings).*

❽ Checklist

8a Please fill in the number of sheets for each of the following types of document contained in this application

Continuation sheets for this Patents Form 1/77 ☐

Claim(s) **2** /
Description **13** /
Abstract
Drawing(s)

8b Which of the following documents also accompanies the application?

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Priority documents *(please state how many)* ☐

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Patents Form 7/77 - Statement of Inventorship and Right to Grant ☐

Patents Form 9/77 - Preliminary Examination Report ☐

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❾ Request

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Signed Vivien West Date: 18 November 1997
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Novel compounds

Background of invention

This invention relates to novel polynucleotides, polypeptides encoded by them and to the use of such polynucleotides and polypeptides, and to their production. The invention further relates to novel vaccines comprising the said polypeptides and to their use in the vaccination of humans at risk from allergy, for example to *Dermatophagoides pteronyssinus* allergen Der p1.

Discussion of prior art

Common allergens present in bee venom, house dust mite emanations and parasite proteins have been found to induce mast cell degranulation, and to stimulate interleukin-4 synthesis and secretion, in the absence of antigen-specific IgE. This non-immunological degranulation by prototypPIC allergens, such as bee venom phospholipase A2 or proteases associated with house dust mite emanations, is critically dependent on enzymatic activity.

Der p 1, the group 1 protease allergen of the house dust mite *Dermatophagoides pteronyssinus,* has been found to cleave the low affinity immunoglobulin (Ig)E Fc receptor (CD23) from the surface of human B lymphocytes. Cleavage of the receptor from the B cell surface was associated with a parallel increase in soluble CD23 in the culture supernatant. The proteolytic effect of Der p 1 is specific for CD23, since none of the other B cell markers tested were affected. It has been speculated that the loss of cell surface CD23 from IgE-secreting B cells may promote and enhance IgE immune responses by ablating an important feedback inhibitory mechanism that normally limits IgE synthesis. Furthermore, since soluble CD23 has been shown to promote IgE production, fragments of CD23 released by Der p 1 may directly

enhance the synthesis of IgE. Thus the proteolytic activity of Der p 1 is mechanistically linked to the potent allergenicity of house dust mites.

Three recent papers have described this work (Machado, D.C., et al. *Eur. J. Immunol.* (1996) 26: 2972-2980; Hewitt, C.R.A., et al *J. Exp. Med.* (1995) 182: 1537-1544; Schulz, O., et al. *Eur. J. Immunol.* (1995) 25: 3191-3194).

Other allergens having proteolytic activity include Der f1 and other allergens of group I (cystein proteases), as well as allergens of groups III (serine proteases) and IV (amylases).

In the context of the immunotherapy of allergy where the objective is to downregulate the production of IgE and maybe to upregulate the production of IgG, as well as to modify the cell mediated response to the allergen, through a shift from a Th2 type to a Th1 type of response, it has never previously been proposed to test the potential of molecules whose catalytic activity has been impaired.

Summary of invention

The present invention provides a mutant allergen having significantly reduced proteolytic activity relative to the wild-type allergen, as well as nucleic acids encoding the same, and its use as an immunotherapeutic agent against allergy. A preferred allergen is the house dust mite allergen Der p1.

Der p1 activity can be impaired by introducing mutations into the cDNA or genomic DNA, either at the active site or at the site of cleavage between the propeptide and the mature molecule.

One aspect of the present invention provides a nucleic acid encoding mutated Der p 1 as set out above, and a further aspect of the invention provides mutated Der p 1 *per se*.

A still further aspect of the invention provides a process for the preparation of a mutated Der p1 protein, which process comprises expressing DNA encoding the said protein in a recombinant host cell and recovering the product.

A DNA molecule encoding a mutated Der p1 (or other mutated allergen) forms a further aspect of the invention and can be synthesized by standard DNA synthesis techniques, such as by enzymatic ligation as described by D.M. Roberts et al in Biochemistry 1985, 24, 5090-5098, by chemical synthesis, by in vitro enzymatic polymerization, or by a combination of these techniques.

Enzymatic polymerisation of DNA may be carried out in vitro using a DNA polymerase such as DNA polymerase I (Klenow fragment) in an appropriate buffer containing the nucleoside triphosphates dATP, dCTP, dGTP and dTTP as required at a temperature of 10°-37°C, generally in a volume of 50µl or less. Enzymatic ligation of DNA fragments may be carried out using a DNA ligase such as T4 DNA ligase in an appropriate buffer, such as 0.05M Tris (pH 7.4), 0.01M $MgCl_2$, 0.01M dithiothreitol, 1mM spermidine, 1mM ATP and 0.1mg/ml bovine serum albumin, at a temperature of 4°C to ambient, generally in a volume of 50µl or less. The chemical synthesis of the DNA polymer or fragments may be carried out by conventional phosphotriester, phosphite or phosphoramidite chemistry, using solid phase techniques such as those described in 'Chemical and Enzymatic Synthesis of Gene Fragments - A Laboratory Manual' (ed. H.G. Gassen and A. Lang), Verlag Chemie, Weinheim (1982),or in other scientific publications, for example M.J. Gait, H.W.D. Matthes, M. Singh, B.S. Sproat, and R.C. Titmas, Nucleic Acids Research, 1982, 10, 6243; B.S. Sproat and W. Bannwarth, Tetrahedron Letters, 1983, 24, 5771; M.D. Matteucci and M.H Caruthers, Tetrahedron Letters, 1980, 21, 719; M.D. Matteucci and M.H. Caruthers, Journal of the American Chemical Society, 1981, 103, 3185; S.P. Adams et al., Journal of the American Chemical Society,1983, 105, 661; N.D. Sinha, J.

Biernat, J. McMannus, and H. Koester, Nucleic Acids Research, 1984, 12, 4539; and H.W.D. Matthes et al., EMBO Journal, 1984, 3, 801.

Alternatively, the coding sequence can be derived from Der p 1 mRNA, using known techniques (e.g. reverse transcription of mRNA to generate a complementary cDNA strand), and commercially available cDNA kits.

The invention is not limited to the specifically disclosed sequence, but includes any proteolytic allergen which has been mutated to remove some or all of its proteolytic activity, whilst retaining the ability to stimulate an immune response against the wild-type allergen.

Mutated versions of Der p 1 may be prepared by site-directed mutagenesis of the cDNA which codes for the Der p 1 protein by conventional methods such as those described by G. Winter et al in Nature 1982, 299, 756-758 or by Zoller and Smith 1982; Nucl. Acids Res., 10, 6487-6500, or deletion mutagenesis such as described by Chan and Smith in Nucl. Acids Res., 1984, 12, 2407-2419 or by G. Winter et al in Biochem. Soc. Trans., 1984, 12, 224-225.

The process of the invention may be performed by conventional recombinant techniques such as described in Maniatis et. al., Molecular Cloning - A Laboratory Manual; Cold Spring Harbor, 1982-1989.

In particular, the process may comprise the steps of:

i) preparing a replicable or integrating expression vector capable, in a host cell, of expressing a DNA polymer comprising a nucleotide sequence that encodes the said mutant Der p1 protein;

ii) transforming a host cell with the said vector;

iii) replacing the cysteine residue from the active site with an alanine residue using site directed mutagenesis; or through replacement of a cDNA fragment by a pair of oligonucleotides whose sequence differ from the natural one; or, alternatively, replacing four residues at the junction between the propeptide and the mature enzyme using site directed mutagenesis;

iv) culturing the transformed host cell under conditions permitting expression of the DNA polymer to produce the protein; and

v) recovering the protein.

The term 'transforming' is used herein to mean the introduction of foreign DNA into a host cell by transformation, transfection or infection with an appropriate plasmid or viral vector using e.g. conventional techniques as described in Genetic Engineering; Eds. S.M. Kingsman and A.J. Kingsman; Blackwell Scientific Publications; Oxford, England, 1988. The term 'transformed' or 'transformant' will hereafter apply to the resulting host cell containing and expressing the foreign gene of interest.

The expression vector is novel and also forms part of the invention.

The replicable expression vector may be prepared in accordance with the invention, by cleaving a vector compatible with the host cell to provide a linear DNA segment having an intact replicon, and combining said linear segment with one or more DNA molecules which, together with said linear segment encode the desired product, such as the DNA polymer encoding the Der p 1 protein under ligating conditions.

Thus, the DNA polymer may be preformed or formed during the construction of the vector, as desired.

The choice of vector will be determined in part by the host cell, which may be prokaryotic or eukaryotic. Suitable vectors include plasmids, bacteriophages, cosmids and recombinant viruses.

The preparation of the replicable expression vector may be carried out conventionally with appropriate enzymes for restriction, polymerisation and ligation of the DNA, by procedures described in, for example, Maniatis et al cited above.

The recombinant host cell is prepared, in accordance with the invention, by transforming a host cell with a replicable expression vector of the invention under transforming conditions. Suitable transforming conditions are conventional and are described in, for example, Maniatis et al cited above, or "DNA Cloning" Vol. II, D.M. Glover ed., IRL Press Ltd, 1985.

The choice of transforming conditions is determined by the host cell. Thus, a bacterial host such as E. coli may be treated with a solution of $CaCl_2$ (Cohen et al, Proc. Nat. Acad. Sci., 1973, 69, 2110) or with a solution comprising a mixture of RbCl, $MnCl_2$, potassium acetate and glycerol, and then with 3-[N-morpholino]-propane-sulphonic acid, RbCl and glycerol. Mammalian cells in culture may be transformed by calcium co-precipitation of the vector DNA onto the cells or by lipofection. The invention also extends to a host cell transformed with a replicable expression vector of the invention.

Culturing the transformed host cell under conditions permitting expression of the DNA polymer is carried out conventionally, as described in, for example, Maniatis et al and "DNA Cloning" cited above. Thus, preferably the cell is supplied with nutrient and cultured at a temperature below 45°C.

The product is recovered by conventional methods according to the host cell. Thus, where the host cell is bacterial, such as E. coli it may be lysed

physically, chemically or enzymatically and the protein product isolated from the resulting lysate. Where the host cell is mammalian, the product may generally be isolated from the nutrient medium or from cell free extracts. Conventional protein isolation techniques include selective precipitation, absorption chromatography, and affinity chromatography including a monoclonal antibody affinity column.

Preferably, the host cell is E. coli. Alternatively, the expression may be carried out in insect cells using a suitable vector such as a baculovirus. The novel protein of the invention may also be expressed in yeast cells as described for the CS protein in EP-A-0 278 941.

The vaccine of the invention comprises an immunoprotective amount of the mutated version of the Der p1 (or other) allergenic protein. The term "immunoprotective" refers to the amount necessary to elicit an immune response against a subsequent challenge such that allergic disease is averted or mitigated. In the vaccine of the invention, an aqueous solution of the protein can be used directly. Alternatively, the protein, with or without prior lyophilization, can be mixed or absorbed with any of the various known adjuvants. Such adjuvants include, but are not limited to, aluminium hydroxide, muramyl dipeptide and saponins such as Quil A, 3D-MPL (3Deacylated monophosphoryl lipid A), or TDM. As a further exemplary alternative, the protein can be encapsulated within microparticles such as liposomes. In yet another exemplary alternative, the protein can be conjugated to an immunostimulating macromolecule, such as killed Bordetella or a tetanus toxoid.

Vaccine preparation is generally described in New Trends and Developments in Vaccines, Voller et al. (eds.), University Park Press, Baltimore, Maryland, 1978. Encapsulation within liposomes is described by Fullerton, US Patent 4,235,877. Conjugation of proteins to macromolecules is disclosed, for example, by Likhite, US Patent 4,372,945 and Armor et al., US Patent 4,474,757.

Use of Quil A is disclosed by Dalsgaard et al., Acta Vet Scand, 18:349 (1977).

The amount of the protein of the present invention present in each vaccine dose is selected as an amount which induces an immunoprotective response without significant, adverse side effects in typPICal vaccines. Such amount will vary depending upon which specific immunogen is employed and whether or not the vaccine is adjuvanted. Generally, it is expected that each dose will comprise 1-1000 mg of protein, preferably 1-200 mg. An optimal amount for a particular vaccine can be ascertained by standard studies involving observation of antibody titres and other responses in subjects. Following an initial vaccination, subjects will preferably receive a boost in about 4 weeks, followed by repeated boosts every six months for as long as a risk of disease exists.

A further aspect of the invention provides a method of preventing or mitigating an allergic disease in man, which method comprises administering to a subject in need thereof an immunogenically effective amount of a mutated allergen of the invention, or of a vaccine in accordance with the invention.

The examples which follow are illustrative but not limiting of the invention. Restriction enzymes and other reagents were used substantially in accordance with the vendors' instructions.

Examples

Example I

Construction of pNIV4811

pNIV4811 is designed to promote the expression of mature Der p1 in fusion with the prepropeptide of *Pichia pastoris* MFα

Ligate : - *Sph*I-*Xho*I from pPIC9k
- *Xho*I-*Pst*I oligonucleotides whose sequences follow (n° 97038 and n° 97039)
- *Pst*I-*Xba*I from pNIV4810 (plasmid ATCC98307)
- *Avr*II-*Sph*I from pPIC9k

Sequences of the oligonucleotides:

n° 97038
5'TCGAGAAAAGAGAGGCTGAAGCTACTAACGCCTGCA3'

n° 97039
5'GGCGTTAGTAGCTTCAGCCTCTCTTTTC3'

Construction of pNIV4817

pNIV4817 is derived from pNIV4811.
It is designed to promote the expression of the mature Der p1 in fusion with the prepeptide of *Pichia pastoris* MFα..

Ligate: - *Bst*EII-*Bam*HI from pNIV4811
- *Bam*HI-*Pst*I oligonucleotides n° 97262 and n°97263 whose sequence follows
- *Pst*I-*Bst*EII from pNIV4811

Sequences of the oligonucleotides:

n° 97262

5'GATCCAAACGATGAGATTTCCTTCAATTTTTACTGCAGTTTTATTCGC AGC ATCCTCCGCATTAGCTGCTCCAACTAACGCCTGCA3'

n° 97263
5'GGCGTTAGTTGGAGCTAATGCGGAGGATGCTGCGAATAAAACTG
CAGTAAAAATTGAAGGAAATCTCATCGTTTG3'

Construction of pNIV4819

Starting from pNIV4817, an expression plasmid designed to produce the mature form of Der p1 in *Pichia pastoris*, the following construction is made to replace the cysteine residue from the active site by an alanine residue.

Ligate: - *Bpu*1102I-AseI fragment from pNIV4817
Oligonucleotides (113 and 114 bases) whose sequence follows (n° 97121 and 97122) : residues I_{104} to E_{142} from figure (I_6 of mature protein)
- *Tfi*I-*Bste*EII fragment from pNIV4810 (ATCC 87307)
- *Bst*EII-*Bpu*11021 fragment from pNIV4817

Sequences of the oligonucleotides

n° 97121

5'TAATGGAAATGCTCCAGCTGAAATCGATTTGCGACAAATGCGAACTG
TCACTCCCATTCGTATGCAAGGAGGCTGTGGTTCAGCTTGGGCTTTCTC
TGGTGTTGCCGCAACTG3'

Ala 113 bases

n° 97122

5'ATTCAGTTGCGGCAACACCAGAGAAAGCCCAAAGCTGAACCACAGC
CTCCTTGCATACGAATGGGAGTGACAGTTCGCATTTGTCGCAAATCGA
TTTCAGCTGGAGCATTTCCAT3'

114 bases

Example 2

Construction of pNIV4812

Ligate: - *Hind*III-*Xba*I from pEE14
- *Hind*III-*Pst*I oligonucleotides n°97040 and 97041 whose sequence follows
-*Pst*I-*Xba*I from pNIV4810 (plasmid ATCC 87307)

Sequence of the oligonucleotides:

n° 97040
5'AGCTTACCATGAAAATTGTTTTGGCCATCGCCTCATTGTTGGCATTGA
GCGCTGTTTATGCTCGTACTAACGCCTGCA3'

n° 97041
5'GGCGTTAGTACGAGCATAAACAGCGCTCAATGCCAACAATGAGGCG
ATGGCCAAAACAATTTTCATGGTA3'

Construction of pNIV4814

Starting from pNIV4812, an expression plasmid based on pEE14 designed to produce the mature form of Der p1 in CHOK1, the following construction is made to replace the cysteine residue from the active site by an alanine residue.

Ligate : *Afl*II - *Ase*I fragment from pNIV4812.
Same oligonucleotides as in pNIV4813 construction (No 97121 and 97122)
*Tfi*I - *BstE*II fragment from pNIV4810 (ATCC 87307)
*BstE*II - *Afl*II fragment from pNIV4812.

Construction of pNIV4813 and pNIV4814 was made possible thanks to the discovery that in pNIV4810 the codon encoding isoleucine 6 of the mature protein was ATT instead of ATC as published. This sequence is responsible for the presence of the *Ase*I restriction site.

Example 3

Construction of pNIV4815

Starting from pNIV4811, the following construction is made to delete four residues [N-A-E-T (T is the first residue of the mature protein)] at the junction between the propeptide and the mature enzyme.

Ligate : *Bln*I - *BamH*I fragment from pPIC9k (the vector used for expression in *PPIChia pastoris*)
*BamH*I - *Eae*I fragment from pNIV4811
*Eae*I - *EcoR*I fragment generated by RT-PCR with primers No 97142 and 97143. Residues : A_6 to E_{74}.
*EcoR*I - *Pst*I oligonucleotides whose sequence follows (No 97140 and 97141). Residues : F75 to C102 except N96AET99
*Pst*I - *Xba*I fragment from pNIV4810.

Sequence of the oligonucleotides : allowing the NAET deletion.

No 97140

5'AATTCAAAAACCGATTTTTGATGAGTGCAGAAGCTTTTGAACACCTC
A
AAACTCAATTCGATTTGAACGCCTGCA3' 75 bases

No 97141

5'GGCGTTCAAATCGAATTGAGTTTTGAGGTGTTCAAAAGCTTCTGCAC
T
CATCAAAAATCGGTTTTTG3' 67 bases

RT-PCR Primers

No 97142

5'CATGAAAATTGTTTTGGCCATCGCC3' 25 bases
*Eae*I

No 97143

$^{5'}$CGGTTTTTGAATTCATCCAACGAC$^{3'}$ 24 bases

*EcoR*I

Example 4

Construction of pNIV4816

Starting from pNIV4812, the same deletion as for pNIV4815 will be generated and expressed in CHOK1.

Ligate : *Xba*I - *Afl*II fragment from pEE14
*Afl*II - *Eae*I fragment from pNIV4812
*Eae*I - *EcoR*I fragment generated by RT-PCR using primers No 97142 and 97143
*EcoR*I - *Pst*I oligonucleotides No 97140 and 97141
*Pst*I - *Xba*I fragment from pNIV4810.

Claims:

1. An isolated nucleic acid molecule encoding a mutated version of a proteolytic allergen, characterised in that the mutant has reduced proteolytic activity relative to the wild-type allergen.

2. An isolated nucleic acid molecule according to claim 1, encoding a mutated version of the protein Der p1.

3. An isolated nucleic acid molecule according to claim 2, selected from the group consisting of:
(a) a nucleic acid molecule comprising the sequence as set forth in SEQ ID NO:1 from nucleotide 1 to nucleotide 963 encoding a protein containing a replacement of a cysteine residue by an alanine at the active site;
(b) a nucleic acid molecule comprising the sequence as set forth in SEQ ID NO:2 from nucleotide 1 to nucleotide 963 encoding a protein containing a deletion of four residues (NAET) at the junction between the propeptide and the mature enzyme ;
(c) a nucleic acid molecule capable of hybridizing under moderately stringent conditions to the nucleic acid molecules of (a) or (b); and
(d) a nucleic acid molecule differing from the nucleic acid molecule of (a) or (b) or (c) in codon sequence due to the degeneracy of the genetic code.

4. A recombinant DNA molecule according to any preceding claim.

5. A recombinant DNA molecule according to claim 4, operatively linked to a regulatory element.

6. A recombinant host cell transformed with a recombinant DNA molecule according to claim 4 or 5.

7. A host cell according to claim 6 which is selected from a bacterium, a yeast cell, an insect cell, and a mammalian cell.

8. A protein encoded by a nucleic acid according to any one of claims1 to 5.

9. A protein according to claim 8, comprising Der p1 that has had at least one amino acid change within the original Der p1 sequence either at its active site or at the site of cleavage between the propeptide and the mature molecule.

10. A process for producing a protein according to claim 8 or 9, comprising culturing the recombinant host cell of claim 6 under conditions promoting expression of said protein and recovering same.

11. A vaccine composition comprising a protein according to claim 8 or 9, in a mixture with a suitable carrier.

12. A vaccine composition as claimed in claim 11 additionally comprising other allergens.

13. Use of a protein according to claim 8 or 9, for the manufacture of a vaccine for use in prophylaxis or treatment of allergy.

14. A method of treating or preventing allergy which comprises administering to a subject an amount of a pharmaceutical composition containing a protein according to claim 8 or 9.